

# Astrocytes and Neurosteroids: Metabolism of Pregnenolone and Dehydroepiandrosterone. Regulation by Cell Density

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**Abstract.** The rat central nervous system (CNS) has previously been shown to synthesize pregnenolone (PREG) and convert it to progesterone (PROG) and 7 $\alpha$ -hydroxy-PREG (7 $\alpha$ -OH PREG). Astrocytes, which participate to the regulation of the CNS function, might be involved in the metabolism of neurosteroids. Purified type 1 astrocytes were obtained from fetal rat forebrain with the use of selective culture conditions and were identified by immunostaining with specific antibodies (GFAP<sup>+</sup>, A2B5<sup>-</sup>). They were plated at low, intermediate, or high densities (2.5–5  $\times$  10<sup>5</sup>, 1–2  $\times$  10<sup>6</sup>, or 4–8  $\times$  10<sup>6</sup> cells/dish, respectively) and maintained for 21 d. They were then incubated with <sup>14</sup>C-PREG and <sup>14</sup>C-DHEA for 24 h and the steroids extracted from cells and media were analyzed. Most radioactive derivatives were released into incubation media. Two metabolic pathways were mainly observed. PREG and DHEA were oxidized to PROG and androstenedione (ADIONE), respectively, [3 $\beta$ -hydroxysteroid-dehydrogenase,  $\Delta$ 5 $\rightarrow$ 4 3-ketosteroid-isomerase (3 $\beta$ -HSD) activity], and converted to 7 $\alpha$ -OH PREG and 7 $\alpha$ -OH DHEA, respectively (7 $\alpha$ -hydroxylase activity). After low density plating, the formation of PROG and ADIONE was  $\sim$ 10% of incubated radioactivity, tenfold larger than that of 7 $\alpha$ -hydroxylated metabolites. In contrast, after high density plating, low levels of PROG

and ADIONE were formed, whereas the conversion to either 7 $\alpha$ -OH PREG or 7 $\alpha$ -OH DHEA was  $\geq$ 50%. The results expressed per cell indicated that the 3 $\beta$ -HSD activity was almost completely inhibited at high cell density, in contrast to the 7 $\alpha$ -hydroxylation which was maintained or increased. The pattern of steroid metabolism was related to cell density at the time of measurement and not to an early commitment of cells: when primary cultures were plated at high density (8  $\times$  10<sup>6</sup> cells/dish), then subcultured after several dilutions (3-, 9-, or 27-fold), the 3 $\beta$ -HSD activity was recovered only at low density. Furthermore, when 5  $\times$  10<sup>5</sup> cells were centrifuged and the resulting clusters were plated, 3 $\beta$ -HSD activity was decreased, whereas steroid 7 $\alpha$ -hydroxylation was enhanced. This implies that cell density per se, but neither cell number nor a diffusible factor(s) is involved in the regulation of steroid metabolism. We conclude that astrocytes in culture metabolize PREG and DHEA, and that the metabolic conversions and, therefore, the related enzymatic activities depend on cell-to-cell contacts. Thus, astrocytes can deliver steroids to other cell types in the CNS. The nature of the metabolites formed depends on the state of cell aggregation and may be involved in physiological and/or pathological conditions (repair of CNS injury, astrocytic tumors).

**I**N the brain, astrocytes control in part, extracellular environment and neuron function by their implication in ion transport, neurotransmitter metabolism, and synthesis of neurotrophic factors (review in Manthorpe et al., 1986; Walicke, 1989; Martin, 1992).

Steroid hormones are involved in sexual differentiation of the central nervous system (CNS),<sup>1</sup> feed-back mechanisms, reproductive- and stress-related behavior, and mental activ-

ity. Steroids found in the CNS come from the peripheral glands or are synthesized locally (neurosteroids) (review in Robel et al., 1991). Thus, as in endocrine glands (Lieberman et al., 1984), pregnenolone (PREG) is made from cholesterol in glial cells (oligodendrocytes in particular). Dehydroepiandrosterone (DHEA) is found in the rat brain deprived of endocrine glands, although its origin remains uncertain. Glial cell cultures also convert PREG to PROG and to 3 $\alpha$ -hydroxy-5 $\alpha$ -pregnane-20-one (3 $\alpha$ , 5 $\alpha$ -TH PROG) (Jung-Testas et al., 1989; Kabbadj et al., 1992). Furthermore, PREG and DHEA are metabolized to the corresponding 7 $\alpha$ -OH derivatives by a rat brain microsomal enzyme (Akwa et al., 1992). Steroids act in the brain through several

1. *Abbreviations used in this paper:* ADIONE,  $\Delta$ 4-androstene-3,17-dione; DHEA, dehydroepiandrosterone; GFAP, glial fibrillary acidic protein; NUS, NuSerum; PREG, pregnenolone; PROG, progesterone; 7 $\alpha$ -OH, 7 $\alpha$ -hydroxy; 3 $\beta$ -HSD, 3 $\beta$ -hydroxy steroid-dehydrogenase,  $\Delta$ 5 $\rightarrow$ 4 3-ketosteroid-isomerase; TLC, thin layer chromatography.

mechanisms (Baulieu, 1981), either after binding to classical intracellular receptors or via interaction with membrane receptors such as the  $\gamma$ -aminobutyric acid type A (GABA<sub>A</sub>) receptor. Progesterone (PROG) binds to the intracellular PROG receptor found in particular in the hypothalamus (review in McEwen et al., 1979).  $3\alpha$ ,  $5\alpha$ -TH PROG, known as an anesthetic steroid, binds to GABA<sub>A</sub> receptor and potentiates GABAergic neurotransmission (Majewska et al., 1986). Conversely, PREG and DHEA in their sulfate ester form are antagonists of the GABA activity (Majewska et al., 1988; Majewska et al., 1990). Thus, neuroactive steroids may influence the CNS function as endocrine and paracrine/autocrine regulators.

The present work was aimed at defining the metabolism of PREG and of DHEA by astrocytes. It was found that cultures of astrocytes metabolize PREG and DHEA along two main metabolic pathways, namely the oxidative conversion ( $3\beta$ -HSD) to PROG and ADIONE, respectively, and the  $7\alpha$ -hydroxylation to  $7\alpha$ -OH PREG and  $7\alpha$ -OH DHEA. This metabolism was shown to be regulated by cell density through cell-to-cell contacts.

## Materials and Methods

### Materials

[4]-<sup>14</sup>C-PREG (56 mCi/mmol) was purchased from Amersham (Buckinghamshire, UK) and [4]-<sup>14</sup>C-DHEA (51 mCi/mmol) from New England Nuclear (Boston, MA). PROG was from IBF (Clichy, France). PREG, ADIONE, and poly-DL-ornithine were from Sigma Immunochemicals (St. Louis, MO). Glutamine was from GIBCO BRL (Cergy-Pontoise, France) and  $5\alpha$ -pregnane-3, 20-dione ( $5\alpha$ -DH PROG),  $\Delta^5$ -pregnene- $3\beta$ , 20 $\alpha$ -diol (20 $\alpha$ -DH PREG),  $7\alpha$ - and  $7\beta$ -OH PREG,  $7\alpha$ - and  $7\beta$ -OH DHEA, and  $\Delta^5$ -androstene- $3\beta$ , 17 $\beta$ -diol (ADIOL) were generously donated by Roussel-Uclaf (Romainville, France). The solvents of analytical grade were purchased from Merck (Darmstadt, Germany) and Carlo Erba (Milano, Italy).

### Cell Culture

Pregnant Sprague-Dawley rats (Iffa-credo, L'Arbresle, France) were killed on gestational day 18 (day of insemination = day 1). The uteri were opened and fetuses were separated from the surrounding membranes under sterile conditions. Cerebral hemispheres were dissected free from meninges in Ca<sup>2+</sup>, Mg<sup>2+</sup>-free PBS, pH 7.4, containing 30 mM glucose. Cells were dissociated mechanically in this medium by aspirating the tissue several times using a fire polished glass Pasteur pipette. The cell suspension was then centrifuged at 300 g for 3 min and the pellet was resuspended in 10 ml of serum-free culture medium (SFM) consisting of MEM/Ham's F-12 medium 1:1 (vol/vol) with 2 mM glutamine, 30 mM glucose, 3 mM NaHCO<sub>3</sub>, 5 mM Hepes, 5 U/ml penicillin, and 5  $\mu$ g/ml streptomycin. Cell number was determined using a hemocytometer, and the cell suspension was diluted in SFM containing 10% NuSerum (NUS) (Collaborative Research, Bedford, MA) to achieve the desired cell concentration, ranging from  $2.5 \times 10^4$  to  $8 \times 10^5$  cells/ml. Primary cultures were seeded in 100-mm tissue culture Petri dishes (Falcon Plastics, Cockeysville, MD) pretreated with 1.5  $\mu$ g/ml polyornithine (PORN) at  $2.5 \times 10^5$ ,  $5 \times 10^5$ ,  $1 \times 10^6$ ,  $2 \times 10^6$ ,  $4 \times 10^6$ , or  $8 \times 10^6$  cells/dish, in a final volume of 10 ml SFM-NUS and were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>-95% air. The culture medium was changed on days 7, 12, 17, and 21. For immunocytochemistry, cells were plated onto PORN-coated glass coverslips contained in 100-mm Petri dishes.

In some experiments, primary cultures were seeded with  $5 \times 10^5$  cells that had been centrifuged at 300 g for 3 min, and thus formed clusters which were deposited in the center of the dish. When indicated, cell clusters were seeded within an area delimited by a ring of paraffin. The few cells which grew outside the ring were scraped off before incubation with radioactive substrate.

In other experiments, secondary cultures were established on day 12 from high-density plated cultures ( $8 \times 10^6$  cells/dish). Cells were rinsed with PBS-glucose, treated with 0.125% trypsin (wt/vol) and 2.5 mM EDTA

in PBS, mechanically dissociated using a Pasteur pipette and replated either undiluted or diluted 3-, 9-, or 27-fold.

### Immunocytochemistry

Cultures were observed repeatedly under the phase contrast microscope throughout the culture period. Coverslip cultures from 7 to 21 d duration were rinsed quickly in PBS, fixed with 2% paraformaldehyde in PBS for 30 min, rinsed again in PBS, and permeabilized with 0.1% Triton-X100, or fixed with acetic acid/methanol for 15 min. Immunolabeling was carried out at room temperature using the avidin-biotin-peroxidase method (Vectastain Elite reagents; Vector Laboratories, Burlingame, CA). The mouse mAbs to GFAP (1:1600, Sigma Immunochemicals), myelin basic protein (MBP; 1:150, Boehringer Mannheim, Meylan, France), fibronectin (1:600; Sigma Immunochemicals), and A2B5 (1:100, gift of M. Raff) were used to label the cells. After a preincubation in PBS containing 3% normal horse serum for 20 min, cells were incubated with the appropriate mAb for 2 h, rinsed in PBS for 10 min, and then incubated again with a biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories) diluted 1:200 in PBS for 30 min. After washing, the avidin-biotin-peroxidase complex was applied at a dilution of 1:62 for 30 min. The peroxidase activity was revealed by incubation with 3,3' diamino-benzidine tetrahydrochloride (0.5 mg/ml) (Polysciences, Warrington, PA) in the presence of H<sub>2</sub>O<sub>2</sub> (0.01% in Tris buffer, pH 7.4). In some instances coverslips were counterstained with Giemsa. Giemsa or Azan's staining was performed on other coverslips for cytological observation.

### PREG and DHEA Metabolism

#### INCUBATION OF CELL CULTURES

Cultures seeded at different densities were incubated with 100 nM <sup>14</sup>C-PREG or <sup>14</sup>C-DHEA, in 10 ml of SFM-NUS at 37°C for 24 h. Media were collected and cells were scraped with a rubber policeman in 5 ml of PBS and homogenized with an all-glass potter homogenizer.

Control experiments were carried out with either cells fixed in acetic acid/methanol (1:3, vol/vol) for 30 min, or culture medium alone.

For experiments with carbon monoxide (CO), primary cultures established in 75 cm<sup>2</sup> flask (Falcon Plastics) were bubbled with CO for 30 s, the radioactive substrate was added, and the flasks were stoppered.

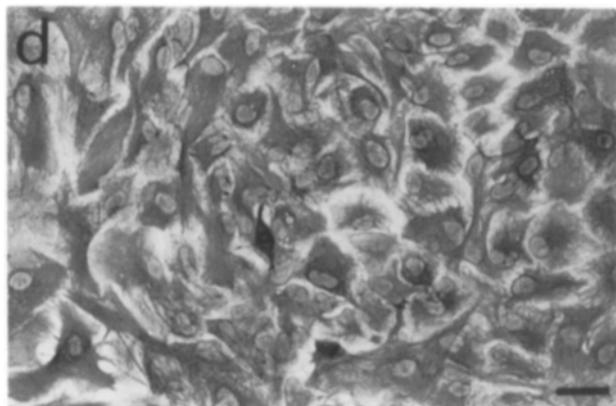
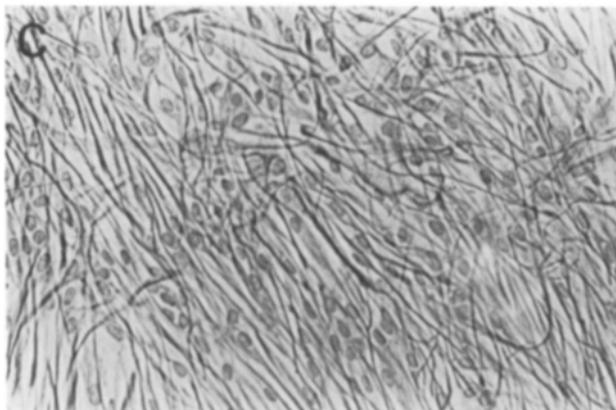
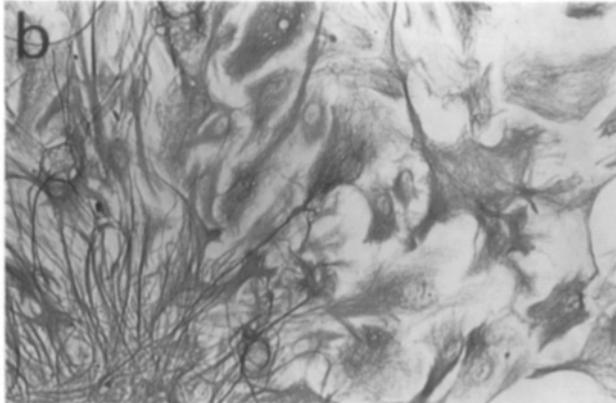
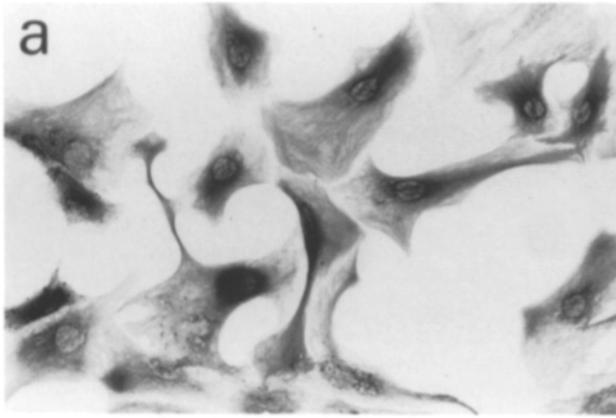
#### RADIOACTIVE STEROID EXTRACTION AND PURIFICATION

Cell homogenates and media were extracted three times with 2 vol of ethyl acetate/isoctane (1:1, vol/vol). The extracts were pooled and taken to dryness in a vacuum centrifuge (Speed-Vac Concentrator, Savant Instruments, Hicksville, NY). Residues were then defatted twice by partition between 90% methanol and isoctane (1:1, vol/vol). Butylated hydroxytoluene (2  $\mu$ g/ml of solvent) was added at every stage of the extraction and purification procedure, and during storage of samples to minimize air oxidation of steroids.

#### CHROMATOGRAPHY OF STEROIDS

**Thin Layer Chromatography.** Extracted steroids were applied on silica gel F254 thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) and developed once in the solvent system chloroform/ethyl acetate (4:1, vol/vol). PROG and ADIONE were used as internal standards and detected under ultraviolet light at 254 nm. The other reference steroids were run on separate lanes and located by spraying with methanol/sulfuric acid (1:1, vol/vol) followed by heating at 100°C for 5 min. The R<sub>f</sub>'s of reference compounds were PREG (0.370  $\pm$  0.009,  $n = 7$ ),  $7\alpha$ -OH PREG (0.029  $\pm$  0.003,  $n = 8$ ),  $7\beta$ -OH PREG (0.048  $\pm$  0.004,  $n = 5$ ), 20 $\alpha$ -DH PREG (0.195  $\pm$  0.008,  $n = 4$ ), PROG (0.543  $\pm$  0.014,  $n = 12$ ),  $5\alpha$ -DH PREG (0.703  $\pm$  0.005,  $n = 6$ ), and DHEA (0.331  $\pm$  0.007,  $n = 8$ ),  $7\alpha$ -OH DHEA (0.016  $\pm$  0.005,  $n = 9$ ),  $7\beta$ -OH DHEA (0.034  $\pm$  0.005,  $n = 5$ ), ADIOL (0.190  $\pm$  0.006,  $n = 5$ ), ADIONE (0.444  $\pm$  0.016,  $n = 12$ ).

**High Pressure Liquid Chromatography.** Reverse phase high pressure liquid chromatography (HPLC) was performed on a 5  $\mu$ m C18 ODS column (4 mm  $\times$  12.5 cm; Merck, Darmstadt, Germany), using methanol-water as the mobile phase, at a flow rate of 1 ml/min. The chromatograph (model 1082 BLC; Hewlett-Packard, les Ulis, France) was equipped with a programmer (79850 BLC terminal) and an automatic injector. Two variants of the elution system were used: system 1, for the identification of PROG and ADIONE, consisted of a linear gradient of 60% to 80% methanol for 30 min. The retention times of reference steroids were: ADIONE,



7–9 min and PROG, 16–18 min. System II, for the identification of  $7\alpha$ -OH PREG and  $7\alpha$ -OH DHEA, consisted of an isocratic mode of 50% methanol for 10 min, followed by a linear gradient of 50 to 100% methanol for 10 min. The retention times of reference steroids were:  $7\alpha$ -OH DHEA, 9–10 min;  $7\alpha$ -OH PREG, 16–17 min. Fractions of 1 ml were collected using a fraction collector (model 202; Gilson France S.A., Villiers Le Bel, France).

#### CRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY

Crystallization of radioactive metabolites was performed after isotopic dilution with appropriate steroid as previously described (Morfin et al., 1973) until constant specific activities of crystals and mother-liquors were reached.

#### RADIOACTIVITY MEASUREMENTS

Quantitation of radioactive areas on TLC plates were made with an automatic TLC linear analyzer (Multitrace Master model LB-285; Berthold Analytical Instruments, Nashua, NH).

Radioactive steroids on TLC plates were detected by autoradiography using Fuji X-ray films (Fuji Photo Film, Co. Ltd, Japan) exposed for 3 d at room temperature. The radioactive areas associated with each metabolite were scraped from the gel and eluted with 5 ml of ethyl acetate.

Radioactivity recovered in cells and media after incubation, extraction, and purification, and from TLC and HPLC fractions was counted in 5 ml of scintillation fluid (Pico-Fluor 15; Packard Instruments, Meriden, CT) with a Tri-carb 4660 spectrometer (Packard Instruments) equipped with quench correction.

#### OTHER MEASUREMENT

Cell DNA content was determined as previously described (Groyer and Robel, 1980).

## Results

### *Type-1 Astrocytes Take Different Shapes According to Cell Density*

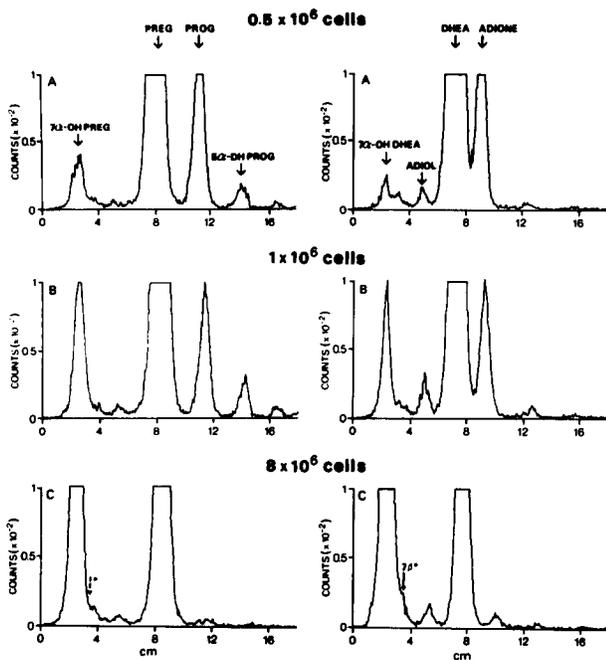
Striking differences in cell shape were observed on day 21 in primary cultures seeded at several densities.

When cells were plated at low density ( $2.5\text{--}5 \times 10^5$ /dish), they formed a dispersed monolayer of large flat cells assuming an epithelial-like morphology (Fig. 1 a). In few areas, small colonies of confluent cells with the same polygonal shape were also present. The cells were GFAP<sup>+</sup> and A2B5<sup>-</sup>, the antigenic phenotype expected from type 1 astrocytes (Raff et al., 1983). Most cells had a dense, perinuclear bundle of stained GFAP<sup>+</sup> filaments extending throughout the cytoplasm; however, some were only slightly stained. No oligodendrocytes (MBP<sup>+</sup> cells) were observed.

When cells were plated at high density ( $4\text{--}8 \times 10^6$ /dish), most of them had one or two long processes displayed in either parallel array or an interwoven network (Fig. 1 c). Some cells were multipolar with densely stained GFAP<sup>+</sup> filamentous material in their stellate processes. All cells were A2B5 negative and cultures were devoid of oligodendrocytes.

When cells were plated at intermediate densities ( $1\text{--}2 \times 10^6$ /dish), the two morphologically distinct types of astro-

*Figure 1.* Astrocytes seeded at various cell densities. (a–c) Primary cultures of cells plated at  $0.5$ ,  $1$ , and  $8 \times 10^6$  per dish, respectively, and grown for 21 d in SFM-NUS. (d) Secondary culture established from  $8 \times 10^6$  plated cells per dish grown in primary culture for 12 d as in c and subcultured at the same density for 10 d. Immunoperoxidase staining with mAb to GFAP and counterstaining with Giemsa. Bar,  $100 \mu\text{m}$ .



**Figure 2.** Radioactive steroids in incubation medium. Cultures of astrocytes, plated at several densities as indicated in A–C, were incubated with  $^{14}\text{C}$ -PREG (left) or  $^{14}\text{C}$ -DHEA (right) in 10 ml of SFM-NUS for 24 h. The extracts of incubation media were submitted to TLC in the solvent system chloroform/ethyl acetate (4:1, vol/vol). Radioactivity was measured with an automatic TLC linear analyzer, as reported in Materials and Methods. When  $7\alpha$ -OH derivatives were formed in high amounts, the  $7\beta$ -OH steroids which are slightly less polar than the  $7\alpha$ -OH isomers, were easily detected on autoradiograms, although in low amounts. They were, however, difficult to visualize on scans at the magnification used in the figure.

cytes related to low or high density cultures were seen. One type or the other prevailed, depending on cell density at seeding (Fig. 1 b).

### Identification of PREG and DHEA Metabolites

Radioactive metabolites were first characterized by one dimensional TLC, scanning (Fig. 2) and autoradiography.

When  $^{14}\text{C}$ -PREG was incubated with astrocytes, five radioactive spots with Rfs of  $7\alpha$ -OH PREG,  $7\beta$ -OH PREG, PROG,  $20\alpha$ -DH PREG, and  $5\alpha$ -DH PROG were located besides PREG on autoradiograms.

When  $^{14}\text{C}$ -DHEA was used as precursor, four radioactive areas with Rfs of  $7\alpha$ -OH DHEA,  $7\beta$ -OH DHEA, ADIONE, and ADIOL were visualized as well as DHEA on autoradiograms.

In each experiment, radioactive areas corresponding to PROG, ADIONE,  $7\alpha$ -OH PREG, and  $7\alpha$ -OH DHEA were eluted. Eluates corresponding to each steroid were pooled and characterized by HPLC. The purity of radioactive compounds was in the 80–99% range. Their identity was confirmed by crystallization after isotopic dilution (Table 1).

### Cell Density-Dependent Metabolism of PREG and DHEA

Primary astroglial cell cultures, seeded at several densities and grown for 21 d in SFM-NUS, were incubated with either  $^{14}\text{C}$ -PREG or  $^{14}\text{C}$ -DHEA at  $37^\circ\text{C}$  for 24 h. Radioactivity

**Table I.** Crystallization after Isotopic Dilution of  $^{14}\text{C}$ -PREG,  $^{14}\text{C}$ - $7\alpha$ -OH PREG,  $^{14}\text{C}$ -ADIONE, and  $^{14}\text{C}$ - $7\alpha$ -OH DHEA Synthesized by Astrocytes

| Metabolite         | Solvent                 | Specific activities |                |           |
|--------------------|-------------------------|---------------------|----------------|-----------|
|                    |                         | Crystals            | Mother liquors | C/M ratio |
| (dpm/mg)           |                         |                     |                |           |
| PREG               | Theoretical             | 1,289               |                |           |
|                    | acetone/isooctane       | 1,224               | 1,426          | 0.86      |
|                    | ethyl acetate/isooctane | 1,273               | 1,376          | 0.93      |
| $7\alpha$ -OH PREG | ethyl acetate/n-hexane  | 1,081               | 1,151          | 0.94      |
|                    | Theoretical             | 3,063               |                |           |
|                    | ethyl acetate/n-hexane  | 2,354               | 2,364          | 0.96      |
| ADIONE             | acetone/n-hexane        | 2,577               | 2,646          | 0.97      |
|                    | ethyl acetate/isooctane | 2,655               | 2,575          | 1.03      |
|                    | Theoretical             | 1,450               |                |           |
| $7\alpha$ -OH DHEA | acetone/isooctane       | 1,452               | 1,770          | 0.82      |
|                    | ethyl acetate/isooctane | 1,448               | 1,554          | 0.93      |
|                    | ethyl acetate/n-hexane  | 1,323               | 1,425          | 0.93      |
| $7\alpha$ -OH DHEA | Theoretical             | 5,662               |                |           |
|                    | ethyl acetate/n-hexane  | 5,074               | 5,895          | 0.86      |
|                    | acetone/n-hexane        | 4,523               | 4,862          | 0.93      |
|                    | ethyl acetate/isooctane | 4,267               | 4,422          | 0.96      |

Astrocytes were incubated with 100 nM  $^{14}\text{C}$ -PREG or  $^{14}\text{C}$ -DHEA. The radioactive metabolites were characterized by TLC and HPLC. Authentic PROG (20.20 mg),  $7\alpha$ -OH PREG (20.78 mg), ADIONE (20.00 mg), and  $7\alpha$ -OH DHEA (20.00 mg) were mixed respectively with 25,917, 63,645, 28,999, and 113,241 dpm of the respective putative  $^{14}\text{C}$ -metabolite.

recovered in the cells was 4–10% of total for PREG and 3–5% for DHEA. For each metabolite, the amounts recovered in incubation medium was  $\sim 20$ -fold larger than in cells.

In cultures plated at low density ( $2.5$ – $5 \times 10^5$  cells/dish) PREG and DHEA were converted to PROG and ADIONE, respectively, in the 10–15% range whereas the formation of  $7\alpha$ -OH derivatives was tenfold smaller (Table II). In contrast, in cultures plated at high density ( $4$ – $8 \times 10^6$  cells/dish), very low amounts of PROG and ADIONE were detected ( $<0.5\%$ ), whereas the conversion to  $7\alpha$ -OH PREG and  $7\alpha$ -OH DHEA was in the 40–70% range. The results expressed in pmole/ $\mu\text{g}$  DNA indicated that the production rate of  $7\alpha$ -hydroxylated metabolites varied little according to cell density, although it tended to increase at the highest densities (Fig. 3).

Other metabolites of PREG and DHEA were found (Fig. 2).  $7\beta$ -OH PREG and  $7\beta$ -OH DHEA were observed either at intermediate or high densities. The conversion rate was in the 7–10% range, three- to ninefold lower than that of the corresponding  $7\alpha$ -OH derivatives. The production of  $5\alpha$ -DH PROG or ADIOL observed after incubation with PREG or DHEA, respectively, was  $\sim 1\%$  of incubated radioactivity. Only trace amounts of  $20\alpha$ -DH PREG were detected.

In control experiments, after incubation of fixed cells or of culture medium alone, the background radioactivity eluted at the position of  $7\alpha$ -OH PREG,  $7\alpha$ -OH DHEA, PROG, and ADIONE did not exceed 1% of incubated radioactivity.

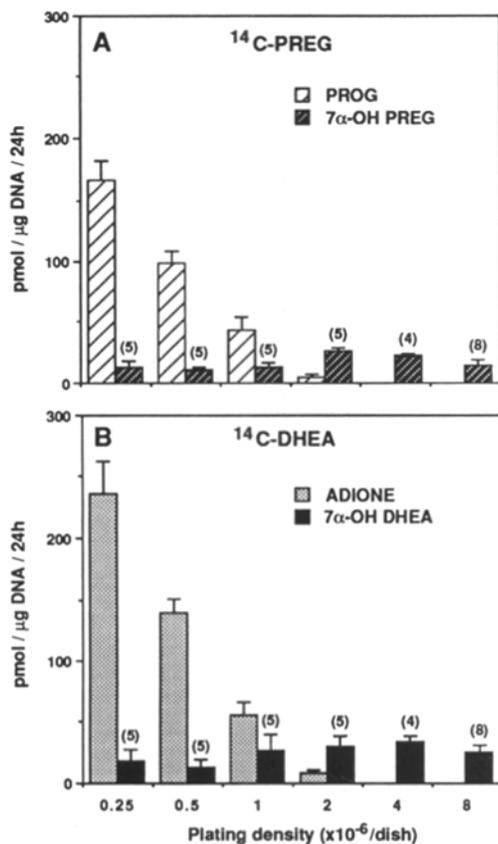
### Inhibition of $7\alpha$ -hydroxylation by Carbon Monoxide

When astrocytes seeded either at high or intermediate den-

**Table II. Radioactive Steroids in Incubation Medium after TLC Analysis**

| Plating density       | Percent of incubated radioactivity (mean ± SEM) |             |                     |            |             |                     |
|-----------------------|---|-------------|---------------------|------------|-------------|---------------------|
|                       | PREG  | PROG        | 7 $\alpha$ -OH PREG | DHEA       | ADIONE      | 7 $\alpha$ -OH DHEA |
| (n)                   |   |             |                     |            |             |                     |
| 2.5 × 10 <sup>5</sup> | 71.9 ± 4.3                                      | 9.9 ± 1.0   | 0.8 ± 0.3           | 77.1 ± 5.9 | 14.2 ± 1.6  | 1.1 ± 0.5           |
| (5)                   |   |             |                     |            |             |                     |
| 5 × 10 <sup>5</sup>   | 68.2 ± 1.9                                      | 10.6 ± 1.0  | 1.4 ± 0.3           | 75.1 ± 2.4 | 14.0 ± 1.1  | 1.4 ± 0.5           |
| (5)                   |   |             |                     |            |             |                     |
| 1 × 10 <sup>6</sup>   | 62.8 ± 2.3                                      | 9.6 ± 2.3   | 2.9 ± 0.9           | 72.2 ± 8.6 | 12.1 ± 2.4  | 5.9 ± 2.9           |
| (5)                   |   |             |                     |            |             |                     |
| 2 × 10 <sup>6</sup>   | 39.2 ± 5.8                                      | 5.3 ± 2.0   | 25.7 ± 6.3          | 34 ± 14    | 9.7 ± 2.1   | 33.7 ± 9.3          |
| (5)                   |   |             |                     |            |             |                     |
| 4 × 10 <sup>6</sup>   | 21.8 ± 6.7                                      | 0.62 ± 0.06 | 47.5 ± 3.5          | 5.7 ± 2.8  | 0.62 ± 0.3  | 71.8 ± 9.7          |
| (6)                   |   |             |                     |            |             |                     |
| 8 × 10 <sup>6</sup>   | 34.8 ± 9.3                                      | 0.28 ± 0.05 | 35.8 ± 9.9          | 18 ± 9     | 0.42 ± 0.17 | 61.8 ± 12.4         |
| (8)                   |   |             |                     |            |             |                     |

Astrocytes were grown at several densities in primary cultures and incubated with 100 nM <sup>14</sup>C-PREG or <sup>14</sup>C-DHEA in 10 ml of SFM-NUS for 24 h. Radioactive steroids extracted from incubation media were analyzed by TLC and quantitated with the Multitrac Master. The data are expressed as percent of radioactivity (mean ± SEM). n, number of experiments.

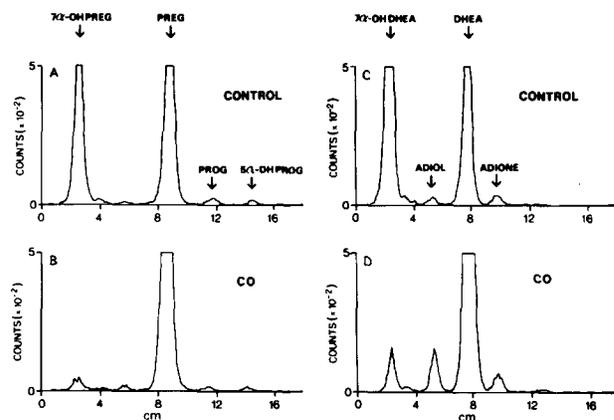


**Figure 3.** Metabolism of PREG and DHEA by primary cultures of astrocytes depends on cell density. Cells plated at different densities and grown for 21 d were incubated with 100 nM <sup>14</sup>C-PREG (A) or <sup>14</sup>C-DHEA (B) in 10 ml of SFM-NUS at 37°C for 24 h. Radioactive steroids were extracted from media and analyzed by TLC. Metabolic conversion rates are expressed in pmoles of product formed per μg DNA per 24 h. Bars indicate the mean ± SEM. ( ) number of experiments.

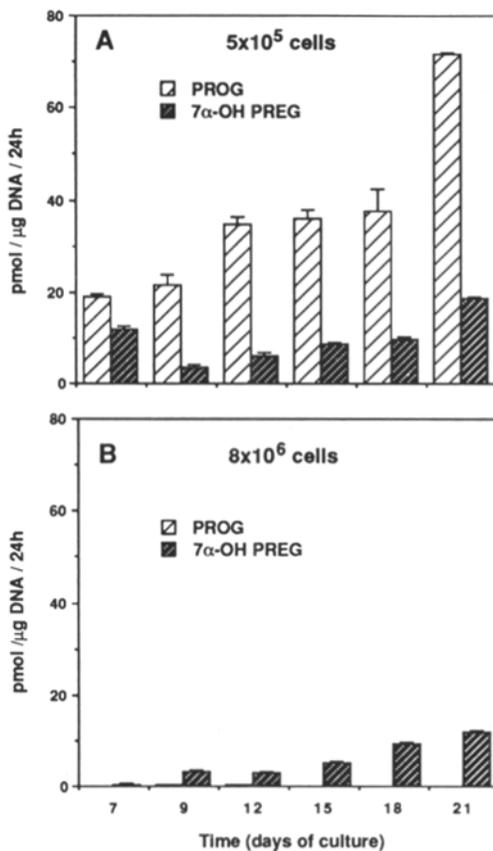
sity were bubbled with CO and incubated with <sup>14</sup>C-PREG or <sup>14</sup>C-DHEA, the production of 7 $\alpha$ -OH PREG and 7 $\alpha$ -OH DHEA greatly decreased (by 83–97%) compared to control (Fig. 4). As expected, the formation of PROG, 5 $\alpha$ -DH PROG, and ADIOL was not inhibited and the production of ADIOL was even increased. The specific inhibitory effect of CO on the 7 $\alpha$ -hydroxylation suggests that a cytochrome P450 is involved in this reaction.

#### Time Course of PREG Metabolism

PREG metabolism was analyzed at different stages in cul-



**Figure 4.** Metabolism of PREG and DHEA by primary cultures of astrocytes after exposure to carbon monoxide. Cells seeded at 8 × 10<sup>6</sup> cells/dish were incubated on day 21 with 100 nM <sup>14</sup>C-PREG (A) or <sup>14</sup>C-DHEA (C) in 10 ml of SFM-NUS for 24 h as usual, or under an atmosphere of CO (B and D) as described in Materials and Methods. Steroids were extracted from the media and analyzed by TLC using chloroform/ethyl acetate 4:1 (vol/vol) as the solvent system. Radioactivity was measured with an automatic TLC linear analyzer.



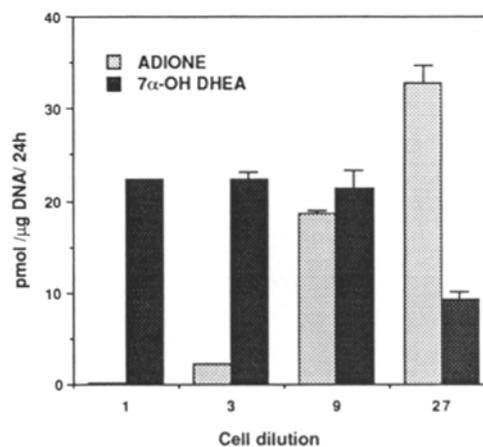
**Figure 5.** Time course of PROG and 7 $\alpha$ -OH PREG synthesis by astrocytes. Primary cultures, seeded at  $5 \times 10^5$  (A) and  $8 \times 10^6$  (B) cells per dish, were incubated at the indicated time intervals with 100 nM  $^{14}\text{C}$ -PREG in SFM-NUS at 37°C for 24 h. Radioactive steroids were extracted from media and analyzed by TLC. Rates of steroid synthesis are expressed in pmoles of product formed per  $\mu\text{g}$  DNA per 24 h (mean  $\pm$  SEM;  $n = 3$ ).

tures seeded at low ( $5 \times 10^5$ ) or high ( $8 \times 10^6$ /dish) density. PROG formation increased from day 7 to day 21 at low density (Fig. 5 A), whereas it remained at its lowest level throughout the culture period at high density (Fig. 5 B). The production of 7 $\alpha$ -OH PREG increased from day 9 to day 21, at low as well as high density. Therefore, the ability of astrocytes to metabolize PREG increased with time during the three week culture period. On the other hand, the inhibitory effect of high cell density on PROG synthesis from PREG was independent of the age of the culture.

#### **Steroid Metabolism Is Not Related to Irreversible Cell Commitment**

To determine whether the astrocytes plated at high density were irreversibly committed during the initial period of culture to cease the formation of  $\Delta 4$ -3-ketosteroids, cells were seeded at  $8 \times 10^6$ /dish, grown for 12 d, and then transferred to secondary culture at various dilutions.

When the cells were replated undiluted, and grown for 10 more days in secondary culture, their shape was of the epithelial-like type (Fig. 1 d). DHEA metabolism resembled that of the high density primary cultures, with a high yield of 7 $\alpha$ -OH DHEA and a complete inhibition of ADIONE production (Fig. 6).



**Figure 6.** Metabolism of DHEA by secondary cultures of astrocytes depends on cell density. Primary cultures plated at high density ( $8 \times 10^6$  cells/dish) were subcultured on day 12 at different dilutions (1, 3, 9, and 27) and grown for 10 d. Incubations were carried out with 100 nM  $^{14}\text{C}$ -DHEA in 10 ml of SFM-NUS at 37°C for 24 h. Radioactive steroids were extracted from media and analyzed by TLC. Metabolic conversion rates are expressed in pmoles of product formed per  $\mu\text{g}$  DNA per 24 h (mean  $\pm$  SEM;  $n = 3$ ).

When astrocytes were diluted 3-, 9-, or 27-fold, the rate of ADIONE formation increased with cell dilution. 7 $\alpha$ -OH DHEA was synthesized at all densities with a decreased rate at the highest dilution. In cultures diluted 3-, 9-, or 27-fold, DHEA metabolism was similar to that of primary cultures plated at 2, 1, or 0.25–0.5  $\times 10^6$  cells/dish, respectively.

Thus, the inhibition of ADIONE formation in high density primary cultures can be reversed, provided the astrocytes are placed secondarily in low cell density conditions.

#### **Cell Morphology Does Not Correlate with Steroid Metabolism**

As we have observed striking differences in the shapes of astrocytes according to cell plating density, the relationship between cell morphology and steroid metabolism was considered. Indeed, at low cell density, astrocytes were epithelial-like and the steroid metabolism pathway was mainly oriented towards the production of PROG and ADIONE, whereas in high density cultures, cells had a fibrous appearance and the 3 $\beta$ -HSD activity was inhibited. However, a complete inhibition of the production of ADIONE was observed in the undiluted secondary culture (Fig. 6), although the cell shape was of the epithelial type (Fig. 1 d).

#### **Cell Compaction Determines Steroid Metabolism**

To investigate which type of interaction between cells was responsible for the regulation of steroid metabolism, the influence of plating conditions on the metabolism of DHEA was further examined by seeding  $5 \times 10^5$  cells/dish, either directly dispersed (control) as usual, or after formation of clusters by centrifugation.

Clusters tended to persist throughout the culture period. After 21 d (Fig. 7), cells in the aggregates were trapped within a network of thin GFAP<sup>+</sup> processes. Close to the cluster, cells had one or several processes displayed in a radial disposition. Farther, cells were large, flat and polygonal. Between these two zones, some astrocytes retained an



**Figure 7.** Astrocytes seeded after centrifugation. Cells ( $5 \times 10^5$  per dish) were centrifuged (300 g for 3 min) and then seeded in SFM-NUS. Two types of cell shapes were observed at day 21: cells with a fibrous appearance around the cluster and epithelial-like astrocytes at the periphery. At the border between the two zones, astrocytes with intermediate shapes (epithelioid with processes) were seen (arrow). Immunoperoxidase staining with mAb against GFAP and counterstaining with Giemsa. Bar, 200  $\mu$ m.

epithelioid shape, with processes oriented towards the cluster (see arrow in Fig. 7). In such cultures (compacted + dispersed), the production of ADIONE from DHEA was half that of the control dispersed cultures, whereas the formation of  $7\alpha$ -OH DHEA was increased (Fig. 8).

To confirm that cell aggregation was responsible for the decrease of ADIONE production, cells obtained after centrifugation were seeded inside a ring of paraffin and the peripheral scattered cells scraped off. The production of ADIONE by the compacted cells was even much smaller, whereas that of  $7\alpha$ -OH DHEA was maintained (Fig. 8). Therefore, it was likely that in the compacted + dispersed cultures (Fig. 7), clustered cells in the central area did not display  $3\beta$ -HSD activity, while isolated cells at the periphery preferentially metabolized DHEA to ADIONE.

## Discussion

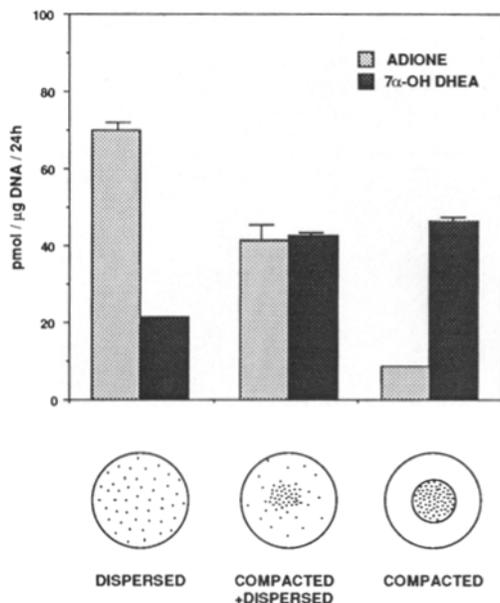
Since the initial observation of PREG and DHEA accumulation in the rat brain, independent of peripheral steroidogenic glands (Corpéchet et al., 1981, 1983), their biological function in the brain has become an active field of investigation. These steroids can be active by themselves or after conversion into metabolites and thus be involved in autocrine or paracrine effects.

It was previously reported that PREG can be converted to PROG in cultures of mixed glial cells (mostly, oligodendrocytes) isolated from newborn rat cerebral hemispheres (Jung-Testas et al., 1989) and in astroglial cell cultures from selective brain areas (that is, cerebral cortex and striatum) of embryonic mouse and rat (Kabbadj et al., 1992). The present work confirms this oxidative pathway in purified cultures of rat fetal forebrain astrocytes, and extends it to the conversion of DHEA to ADIONE. A cytochrome P-450  $7\alpha$ -hydroxylase activity was previously demonstrated in rat brain microsomes (Warner et al., 1989; Akwa et al., 1992).

However, the cell type(s) responsible for this conversion was (were) not defined. Here we show that  $7\alpha$ -hydroxylase activity is present in one type of glial cells, namely, astrocytes. Thus astrocytes possess both  $7\alpha$ -hydroxylase and  $3\beta$ -HSD activities, as definitely demonstrated by identification of the major metabolites of PREG and DHEA by TLC, reverse phase HPLC, and crystallization to constant specific activity.

In the course of this work, we have discovered that the pattern of PREG and DHEA metabolism towards oxidation and hydroxylation was dependent on cell density. When astrocytes were plated at low density, PREG and DHEA were mainly converted to PROG and ADIONE, respectively. When they were plated at high density, either  $7\alpha$ -OH PREG or  $7\alpha$ -OH DHEA was the major metabolite formed. When astrocytes were seeded at  $5 \times 10^5$  per dish, the conversion of PREG to PROG was  $9.9 \pm 1.0\%$  (mean  $\pm$  SEM,  $n = 5$ ), and the conversion of DHEA to ADIONE was  $14.2 \pm 1.6\%$  ( $n = 5$ ). When they were seeded at  $8 \times 10^6$  per dish, insignificant levels of PROG and ADIONE were found indicating that the  $3\beta$ -HSD activity was almost undetectable. The results expressed in pmoles/ $\mu$ g DNA/24h showed that the  $7\alpha$ -hydroxylase activity of astrocytes was maintained or even slightly increased after high plating densities. Accordingly, the percent conversion of radioactive substrate to the corresponding  $7\alpha$ -hydroxylated metabolite increased markedly after high plating densities reaching  $47.5 \pm 3.5\%$  ( $n = 4$ ) for  $7\alpha$ -OH PREG and  $71.8 \pm 9.7\%$  ( $n = 4$ ) for  $7\alpha$ -OH DHEA.

To determine whether the effect of plating density was reversible, cells seeded at high density were trypsinized, and secondary cultures were made at several dilutions. Since the recovery of  $3\beta$ -HSD activity occurred under the secondary low plating density condition, we concluded that the orientation of steroid metabolism did not result from the commitment of cells according to plating conditions. In addition, we



**Figure 8.** Metabolism of DHEA by primary cultures of astrocytes depends on plating conditions. The cells ( $5 \times 10^5$  per dish) were seeded either directly dispersed, or after centrifugation. Centrifuged cells were seeded either in the center of a dish (*compact + dispersed*) or inside a central area delimited by a ring of paraffin (*compact*) as shown on the scheme below the figure. In the latter condition the very few scattered cells outside the ring were scraped off before incubation with radioactive substrate. Cells were grown up in 10 ml SFM-NUS for 21 d and incubated with 100 nM  $^{14}\text{C}$ -DHEA for 24 h. Radioactive steroids were extracted from the media and analyzed by TLC. Rates of metabolites formed are expressed in pmoles of product per  $\mu\text{g}$  DNA per 24 h (mean  $\pm$  SEM;  $n = 3$ ).

found that cell morphology was not correlated to the pattern of steroid metabolism. When high density plated cultures were replated either at the same original density or after 3-, 9-, or 27-fold dilution, the same epithelioid cell shape was always observed (Fig. 1). However, the pattern of steroid metabolism remained dependent on cell density. Remarkably, in high density replated epithelioid cells, a lack of  $3\beta$ -HSD activity was observed as in filamentous high density plated cells of primary cultures.

We could also exclude the depletion of nutrients or hormones in the culture medium, that might occur at high cell density, because the inhibition of  $3\beta$ -HSD activity was maintained in the time-course experiments, even though the culture medium was renewed periodically.

The type of interaction between cells was then considered. The same low number of cells ( $5 \times 10^5$  per dish) was plated, either directly dispersed or after the formation of clusters by centrifugation. When compacted cells were grown inside a delimited area, the conversion of DHEA to ADIONE was greatly decreased as compared to control dispersed cultures. We concluded that the establishment of cell-to-cell contacts, related to cell density, was very likely responsible for the inhibition of  $3\beta$ -HSD activity. The implication of a diffusible factor(s) produced by the aggregates was excluded: when centrifuged cells were seeded in the center of dishes without paraffin rings, there was a decreasing gradient of cell density from the clusters to the periphery,

and most astrocytes were confluent over a large area around the clusters. In such compacted + dispersed cultures, although the total cell number was about the same, the conversion of DHEA to ADIONE was much smaller than in dispersed ones, but still higher than in compacted delimited cell cultures. If a diffusible factor(s) had been produced by the clusters, the rate of formation of ADIONE would have been similarly low in both types of compacted cell cultures. In fact, scattered cells were responsible for the  $3\beta$ -HSD activity in compacted + dispersed cultures: in the latter condition, when the central aggregated-confluent cells were removed and the scattered peripheral cells incubated with  $^{14}\text{C}$ -DHEA, ADIONE was formed as in the control dispersed cultures (data not shown). In addition, the effect of compaction was confirmed by another experiment in which the  $3\beta$ -HSD activity was not detected after the plating of  $5 \times 10^5$  cells in culture dishes of smaller size (25-mm diam instead of 100 mm as usual) (data not shown).

Other examples of density-dependent regulation have been reported for sterol metabolism (Friedman et al., 1987) and for cell surface  $\gamma$ -glutamyl transpeptidase (Morgenstern et al., 1992) in cultured C6 cells, and the regulation of enzyme activities appeared to depend on the extent of cell-to-cell contacts. Variations of intracellular steroid receptor concentration with cell density have also been reported (Gerschenon et al., 1981; Saad et al., 1981; Nakhla et al., 1984).

The molecular mechanisms of cell-to-cell interactions can be of several types. Astrocytes in culture receive information from their neighbors through specialized junctions (Fischer and Kettenmann, 1985). They are connected through gap junctions and form a syncytium after maturation, allowing long-range signaling (Cornell-Bell et al., 1990; Dermietzel et al., 1991). Other mechanisms include the binding of a cell surface ligand on one cell to its membrane receptor on the other or stimulation by secretion in the narrow intercellular gap where the cells are in contact (Singer, 1992).

There is increasing evidence showing that astrocytes are not merely supporting cells but that they fully interact with neurons in regulating the CNS activity (Kimmelberg and Norenberg, 1989; Dani et al., 1992). Astrocytes not only synthesize PROG, but they can convert it to  $3\alpha$ ,  $5\alpha$ -TH PROG (Kabbadj et al., 1992) which is a positive allosteric modulator of GABAergic neurotransmission (Majewska et al., 1986; Paul and Purdy, 1992). Astrocytes can also provide PROG to neighboring neurons, which also contain the enzymes for the formation of  $3\alpha$ ,  $5\alpha$ -TH PROG (Kabbadj et al., 1992). Moreover, glial cells (oligodendrocytes in particular) (Jung-Testas et al., 1991), as well as some neurons, contain a PROG receptor, which may regulate cell growth and specific protein synthesis. Therefore, by metabolizing PREG to PROG, astrocytes might intervene in the regulation of several brain functions. A biological role for  $7\alpha$ -OH PREG or  $7\alpha$ -OH DHEA in the brain has yet to be determined, but the  $7\alpha$ -hydroxylation could regulate the availability of PREG and DHEA as precursors of neuroactive steroids (Akwa et al., 1992).

It is tempting to discuss the *in vivo* significance of the intriguing results obtained with cells in culture. Glial cells play an important role in the repair of the CNS. It is commonly assumed that cerebral injury in adult rats is followed by a specific glial response. This "reactive astroglia" consists of a proliferation and hyperplasia of astrocytes resulting in the

formation of scars with an elevated level of expression of the glial fibrillary acidic protein and an inhibitory action on axon regrowth (Nieto-Sampedro et al., 1985; Janeczko, 1988). Therefore, it would be interesting to study steroid metabolism in these processes of astrocyte proliferation in response to CNS injury, as well as in primary brain tumors such as astrocytomas. The implication of PROG and 7 $\alpha$ -hydroxylated derivatives of PREG and DHEA in the regulation of such events merits further investigation.

In conclusion, the involvement of astrocytes in the metabolism of PREG and DHEA, which is regulated by cell density, is a new function of these glial cells which may take place in the cross-talk of different cell types in the CNS.

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